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CERTIFICATION REPORT

Certification of a reference material of *Campylobacter coli* and *jejuni* (CNET068 and CNET112) agarose plugs for PFGE: IRMM-313

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Certain commercial equipment, instruments, and materials are identified in this paper to specify adequately the experimental procedure. In no case does such identification imply recommendation or endorsement by the European Commission, nor does it imply that the material or equipment is necessarily the best available for the purpose.

Summary

This report describes the production of IRMM-313, a *Campylobacter coli* and *jenuni* gDNA material certified for the size of the DNA fragments obtained by enzymatic restriction and Pulsed Field Gel Electrophoresis (PFGE). The material was produced following ISO Guide 34:2009 [1].

The CRM was produced from cultures of *Campylobacter coli* CNET068 and *jejuni* CNET112 which were pooled and processed into agarose plugs suited for PFGE. The bacterial cells were lysed as to release the gDNA within the plug.

Between unit-homogeneity and stability during dispatch and storage were assessed in accordance with ISO Guide 35:2006 [2].

The material was characterised by an intercomparison among laboratories of demonstrated competence and complying to ISO/IEC 17025. Technically invalid results were removed but no outlier was eliminated on statistical grounds only. The certified values were obtained by PFGE.

Uncertainties of the certified values were calculated in compliance with the Guide to the Expression of Uncertainty in Measurement (GUM) [3].

The material is intended for quality control and assessment of method performance. As any reference material, it can also be used for control charts or validation studies.

The CRM is available in plastic screw cap vials containing 1 plug suspended in Tris EDTA buffer solution. The minimum amount of sample recommended to be used is ½ plug.

The following values were assigned to IRMM-313:

	DNA fragment sizes		
	Fragment number	Certified value [2] [kb]	Uncertainty [3] [kb]
*Sma*I digested DNA fragments[1]	2	458.8	2.0
	3	351.7	2.4
	4	303.0	2.3
	5	263.2	1.9
	6	188.2	1.2
	7	173.2	1.3
	8	131.1	1.5
	9	114.4	1.2
	10	95.5	1.4
	11	81.2	1.7
	12	54.9	2.2
	13	40.7	1.6

1) As defined by the PFGE procedure described in this report

2) Unweighted mean value of the means of accepted sets of data, each set being obtained in a different laboratory. The assigned values are combinations of agreeing results individually traceable to the lambda DNA (cI857 ind 1 Sam7) of the size markers, the assigned fragment sizes themselves are therefore traceable to the lambda DNA (cI857 ind 1 Sam7).

3) The uncertainty is the expanded uncertainty with a coverage factor k = 2 corresponding to a level of confidence of about 95 % estimated in accordance with ISO/IEC Guide 98-3, Guide to the Expression of Uncertainty in Measurement (GUM:1995), ISO, 2008.

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Glossary

ANOVA	Analysis of Variance
BLAST	Basic Local Alignment Search Tool
BSL	Bio-safety Level
*ceu*E	A gene encoding a protein involved in siderophore transport
CLB	Cell Lysis Buffer
CNET	Campynet
CRM	Certified reference material
DNA	Deoxyribonucleic acid
EC	European Commission
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
EU	European Union
EURL	European Union Reference Laboratory
gDNA	Genomic DNA
GUM	Guide to the Expression of Uncertainty in Measurements
h	hour
IEC	International Electrotechnical Commission
ILC	Interlaboratory comparison
IRMM	Institute for Reference Materials and Measurements of the JRC
ISO	International Organization for Standardization
JRC	Joint Research Centre of the European Commission
k	Coverage factor
kb	Kilobase
SDS	Safety data sheet
n	Number of replicates per unit
N	Number of samples (units) analysed
n.a.	Not applicable
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RE	Restriction Enzyme
PFGE	Pulsed Field Gel Electrophoresis
rpm	Revolutions per minute
s	Standard deviation

$s_{between}$	Standard deviation between groups as obtained from ANOVA; an additional index "rel" is added as appropriate
SI	International System of Units
SID Unit	Standards for Innovation and sustainable Development Unit of the IRMM
SKG	SeaKem® Gold
*Sma*I	restriction enzyme isolated from *Serratia marcescens*
s_{within}	Standard deviation within groups as obtained from ANOVA; an additional index "rel" is added as appropriate
T	Temperature
t	Time
TBE	Buffer containing Tris-Borate and EDTA
TE	Buffer containing Tris and EDTA
Tris	Tris(hydroxymethyl)aminomethane
u	standard uncertainty
U_{CRM}	Expanded uncertainty of the certified value; an additional index "rel" is added as appropriate
UV	Ultra violet
V	Volume

1 Introduction

1.1 Background

Campylobacter is a bacterium that can cause an illness in humans called campylobacteriosis. With over 190000 human cases annually, this disease is the most frequently reported foodborne illness in the European Union (EU). The cost of campylobacteriosis to public health systems and to lost productivity in the EU is estimated by EFSA to be around EUR 2.4 billion a year [4].

EU Directive 2003/99/EC on the monitoring of zoonoses and zoonotic agents obliges the Member States to collect relevant and, where applicable, comparable data of zoonoses, zoonotic agents, antimicrobial resistance and food-borne outbreaks.

PFGE is currently one of the most commonly used bacterial typing techniques for *Campylobacter*. IRMM-313 is a valuable tool that can be used for method validation or as a quality control material for PFGE analysis.

1.2 Choice of the material

IRMM-313 consists of agarose plugs with *Campylobacter coli* and *jejuni* gDNA embedded. A gDNA embedded agarose plug suited for PFGE analysis is the preferred format as PFGE analysis is the main bacterial typing method used for foodborne pathogens such as *Campylobacter*.

1.3 Design of the project

IRMM-313 plugs were produced in house and certified values were obtained through an intercomparison study applying the protocols described in this report.

2 Participants

2.1 Project management and evaluation

Project management and evaluation was under responsibility of the European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE
(accredited to ISO Guide 34 for production of certified reference materials, BELAC No. 268-RM)

2.2 Processing

Processing was performed by the European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE
(accredited to ISO Guide 34 for production of certified reference materials, BELAC No. 268-RM)

2.3 Homogeneity study

Homogeneity testing was performed by the European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE

2.4 Stability study

The stability study was performed by the European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE

2.5 Characterisation

Characterisation was performed through an intercomparison, the participating laboratories were:

- Animal Health and Veterinary Laboratory Agency, Surrey, UK
- Anses Laboratoire de Ploufragan-Plouzané, Ploufragan, France
- Faculty of Veterinary Medicine, Food Institute, Skopje, Macedonia
- National Veterinary Institute, Uppsala, Sweden
- National Veterinary Research Institute, Pulawy, Poland
- Netherlands Food and Consumer Product Safety Authority, Wageningen, Netherlands
- State Veterinary and Food Institute, Dolný Kubín, Slovakia
- European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, Belgium

3 Material processing and process control

3.1 Origin of the starting material

Campylobacter coli CNET068 and *Campylobacter jejuni* CNET112 cultures were kindly provided by the National Veterinary Institute, Uppsala, Sweden, the EURL for *Campylobacter*.

3.2 Processing

The *Campylobacter coli* CNET068 and *Campylobacter jejuni* CNET112 cultures were used to produce stocks which were used for the preparation of the PFGE plugs.

The processing steps for the production of IRMM-313 were:

Plug preparation

- *Campylobacter coli* and *jejuni* were cultured on *Campylobacter* selective agar under micro aerobic conditions (10 % CO_2; 5 % H_2; 85 % N_2) for 24 h to 48 h at 42 ºC.

- For each strain, the cells were scraped from the surface of the agar plates using a polyester fibre swab moistened with sterile PBS (0.01 M phosphate buffered saline, pH 7.4) solution and suspended in 50 mL PBS solution to reach an absorbance of ~ 0.820 at a wavelength of 600 nm.

- 50 mL cell suspension of each strain was added together and mixed by inverting the tube 3 times.

- In a 1.5 mL tube 25 µL Proteinase K (20 mg/mL) was gently mixed with 500 µL cell suspension using a 1000 µL pipet tip.

- 500 µL melted 1 % SeaKem® Gold (SKG) agarose at 55 to 60 ºC was added and mixed gently by pipetting up and down 3 times maximum.

- The suspension was dispensed in plug moulds (100 µL/mould) and allowed to solidify at room temperature for 10 to 15 min.

- Previous steps were repeated until no more cell suspension was left.

Cell lysis

- A maximum of 50 plugs were transferred to a 50 mL tube containing 30 mL Cell Lysis Buffer (CLB) (50 mM Tris; 50 mM EDTA, pH 8.0; 1 % Sarcosyl), containing Proteinase K (0.1 mg/mL).

- The tubes were incubated for 15 up to 30 min at 54 ºC in an orbital shaking water bath with shaking between 175 and 200 rpm. The water level in the bath was assured to be above the level of the liquid in the tubes.

Washing

- The lysis mix was carefully poured off and discarded and 30 mL pre-warmed sterile Type I water was added to rinse the plugs.

- The liquid was poured off and discarded and again 30 mL pre-warmed sterile Type I water was added. The tubes were incubated at 54 ºC for 10 to 15 min with orbital shaking between 175 and 200 rpm.

- The liquid was poured off and discarded and 30 mL pre-heated sterile TE (10 mM Tris; 1 mM EDTA, pH 8.0) buffer was added. The tubes were incubated at 54 ºC for 10 to 15 min with orbital shaking between 175 and 200 rpm.

- The previous step was repeated two more times.

- After the last wash, the TE was poured off and discarded and 30 mL fresh, sterile TE was added at room temperature.

- Each plug was transferred to 1.7 mL sterile TE in a 3 mL plastic vial.

- The samples were stored at 4 ºC.

3.3 Process control

The following process controls were carried out to assure the quality of the end product.

3.3.1 Identity confirmation

The identity of *Campylobacter coli* and *Campylobacter jejuni* was confirmed by DNA sequencing of the nucleotide sequence of the *ceu*E gene encoding part of a protein involved in siderophore transport, a putative virulence determinant [5]. Partial between species sequence identity of the *ceu*E gene allows for the design of species-specific primers to distinguish between species. The primers used can be found in Table 1.

Table 1: PCR primers designed complementary to *ceu*E gene regions containing species-specific sequence differences.

Name	Description	sequence
P-0577	*C. jejuni*, *ceu*E gene forward primer	5' CCTGCTACGGTGAAAGTTTTGC 3'
P-0578	*C. jejuni*, *ceu*E gene reverse primer	5' GATCTTTTTGTTTTGTGCTGC 3'
P-0900	*C. coli, ceu*E gene forward primer	5' ATGAAAAAATATTTAGTTTTTGCA 3'
P-0901	*C. coli, ceu*E gene reverse primer	5' ATTTTATTATTTGTAGCAGCG 3'

The obtained sequences were compared to sequences in the GenBank using a BLAST (version 2.2.28+) search.

BLAST is a computer algorithm that is available for online use on the National Center for Biotechnology Information (NCBI) website. The Basic Local Alignment Search Tool (BLAST) is designed to find regions of local similarity between sequences. The program compares nucleotide sequences to sequence databases in the GenBank and calculates the statistical significance of matches [6].

The BLAST search of the sequence obtained from the *C. coli* CNET068 stock and IRMM-313 plugs using *C. coli* specific primers resulted in the same 6 hits covering a 93 to 99 % match to sequences all coding for the *C. coli ceu*E gene or part of the gene.

The BLAST search of the sequence amplified from the *C. jejuni* CNET112 stock and IRMM-313 plugs using *C. jejuni* specific primers resulted in 31 hits covering a 92 to 100 % match to sequences all coding for the *C. jejuni ceu*E gene or part of the gene.

The identity of *C. coli* and *C. jejuni* could be confirmed up to a species level. It was not possible however to find a match with CNET068 and CNET112 strain specific sequences as the sequences of these particular strains are not published in the GenBank.

BLAST search and sequence alignment results can be found in Annex A.

3.3.2 Viability testing

To confirm complete lysis of the bacteria embedded in the IRMM-313 agarose plugs during processing, viability of the bacteria in the plugs was tested according to ISO 10272-1: Microbiology of food and animal feeding stuffs – Horizontal method for detection and enumeration of *Campylobacter* spp.

No growth was detected on the agars inoculated with IRMM-313 plugs.

3.4 Analysis

All PFGE analysis for homogeneity, stability and batch characterisation were performed according to the CampyNet protocol, with minor adjustments, described in this section.

Restriction enzyme digestion

- The plug was cut in 2 equal pieces with a scalpel.

- ½ plug was incubated in 200 µL 1x restriction enzyme buffer solution, as recommended by the manufacturer, at room temperature for 15 min.

- The buffer was removed and discarded using a pipette and 200 µL RE mix, as recommended by the manufacturer, containing 40 units of *Sma*I restriction enzyme was added and mixed by tapping gently.

- The plug was incubated at 25 ºC for 4 h.

PFGE

- 0.5x TBE (44.5 mM Tris-Borate; 1 mM EDTA, pH 8.3) buffer was prepared from 5x TBE buffer by dilution in Type I water.

- The PFGE chamber was filled with sufficient 0.5x TBE buffer, air bubbles trapped in the tubing were removed, the cooler was set at 14 °C and the buffer was circulated for 1 h before use.

- A 150 mL 1 % SKG agarose was prepared in 0.5x TBE and kept at 50 ºC until use.

- The plug pieces were placed onto the teeth of a 30 well comb as far to the bottom as possible. As much buffer as possible was removed. Between every two plugs size marker D2291 and D2416 (Sigma) were loaded.

> D2291 Pulse Marker 0.1–200 kb: 194.0; 145.5; 97.0; 48.5; 23.1; 9.4; 6.6; 4.4; 2.3; 2.0; 0.6; 0.1 (kb)
>
> D2416 Pulse Marker 50-1,000 kb; 727.5; 679.0; 630.5; 582.0; 533.5; 485.0; 436.5; 388.0; ;339.5; 291.0; 242.5; 194.0; 145.5; 97.0; 48.5 (kb).
>
> 48.5 kb being the fragment giving the brightest signal.

- The plugs were fixed onto the comb by pipetting a few drops of 1 % SKG agarose around the plugs.

- The casting stand was assembled and placed on a levelling table. It is important that the casting stand is completely horizontal to achieve the same gel thickness all over the gel.

- The comb with the plugs attached was put in place.

- The 1 % SKG agarose was poured in, making sure not to move the plugs. Any air bubbles were removed. The gel was allowed to solidify for 30 to 45 min.

- The comb was carefully removed from the gel.

- The resulting wells were sealed with 1 % SKG agarose.

- The gel and the black plate were taken out of the casting stand and any agarose was removed from the bottom of the plate.

- The plate with the gel was placed in the electrophoresis chamber, making sure that it fitted tightly in the frame. The chamber was closed, air bubbles trapped in the tubing were removed and it was checked that the cooler was working.

- The run was started with running conditions: Initial switch time: 0.5 s
Final switch time: 40 s
Gradient: 5 V/cm
Angle: 120º
Run time: 22.5 h

- The gel was stained 30 min in 400 mL 1 µg/mL ethidium bromide in Type I water.
- De-staining was done for 2x 20 min in 400 mL Type I water.
- The gel was photographed above a UV-source using a UV orange filter.
- The gel was analysed using dedicated software.

4 Homogeneity

A key requirement for any reference material is the equivalence between the various units. The between unit homogeneity was assessed qualitatively by visual inspection of the band pattern covered in the between-unit homogeneity study.

Within-unit homogeneity was assessed to determine the minimum sample intake.

4.1 Between-unit homogeneity

The between-unit homogeneity was evaluated to ensure that the certified values of the CRM are valid for all units of the material, within the stated uncertainty.

The number of selected units for assessing between-unit homogeneity corresponds to approximately the cubic root of the total number of the produced units. 10 units were selected using a random stratified sampling scheme covering the whole batch for the between-unit homogeneity test. For this, the batch was divided into 10 groups (with a similar number of units) and one unit was selected randomly from each group. Each selected unit was cut in 2 pieces, and each piece was analysed by PFGE. The measurements were performed under repeatability conditions and in a randomised manner. The results are shown in Figure 1.

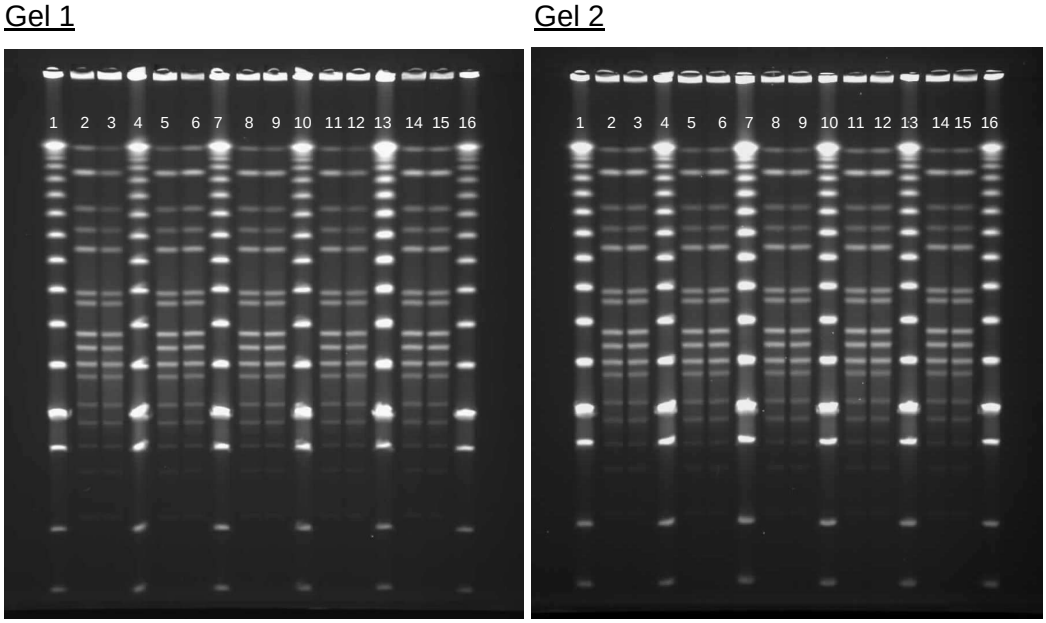

Figure 1: PFGE pattern of size markers and homogeneity samples, obtained by PFGE analysis as described in section 3.4. Lanes 1, 4, 7, 10, 13, 16: size marker D2291 and D2416 (Sigma). Lanes 2, 3, 5, 6, 8, 9, 11, 12, 14, 15 correspond to a series of sample numbers in the table in Annex B.

PFGE analysis of the homogeneity samples showed identical band patterns for all units. i.e. no bands were missing, no new bands appeared and no obvious differences in the resolution of the bands were observed. Therefore the batch was found to be homogenous.

4.2 Minimum sample intake

The minimum sample intake is the minimum amount of sample that is representative for the whole unit and thus can be used in an analysis.

The requirement for this CRM format is that the minimum sample intake still allows for all fragments to be visualised for values to be assigned.

Based on the intercomparison measurements the recommended minimum sample intake allowing visualisation and band assignment of all fragments of the pattern is set at ½ plug. It is recommended to use each part of the plug on one gel.

5 Stability

Stability testing is necessary to establish conditions for storage (long-term stability) as well as conditions for dispatch to the customers (short-term stability).

The stability studies were carried out using an isochronous design [7]. In that approach, samples are stored for a certain time at different temperature conditions. Afterwards, the samples are moved to conditions where further DNA degradation can be assumed to be negligible (reference conditions). At the end of the isochronous storage, the samples are analysed simultaneously under repeatability conditions.

5.1 Short-term stability study

For the short-term stability study, units were stored at 4 °C and 18 °C for 0, 1, 2 and 4 weeks (at each temperature). Additionally 3 units were stored at -70 °C for 1 week to assess the effect of freezing on the plugs. The reference temperature was set to 4 °C. 6 units per storage time were selected using a random stratified sampling scheme. From each time and temperature point, 3 units were measured by PFGE in duplicate. The measurements were performed under repeatability conditions, in a random sequence.

The results of the short-term stability measurements are shown in Figure 2.

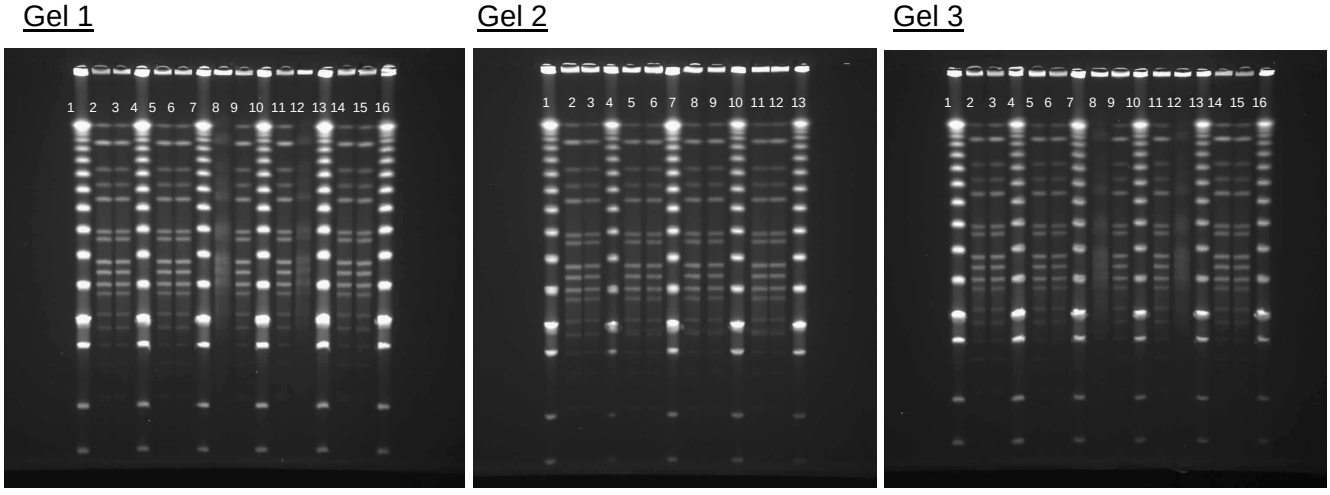

Figure 2: PFGE pattern of size markers and short-term stability samples, obtained by PFGE analysis as described in section 3.4. Lanes 1, 4, 7, 10, 13, 16: size marker D2291 and D2416 (Sigma). Lanes 2, 3, 5, 6, 8, 9, 11, 12, 14, 15 correspond to a series of sample numbers in the table in Annex B. Lanes 8 and 12 of gel 1 and 3 correspond to samples stored at -70 °C.

PFGE analysis of the short-term stability samples showed identical band patterns for all units stored at 4 ºC and 18 ºC. i.e. no bands were missing, no new bands appeared and no

smears or obvious differences in the resolution of the bands were observed. Partial to complete degradation was observed at -70 °C, no fragment sizes could be assigned.

It can be concluded that the batch will be stable during dispatch up to 18 ºC. The material shall therefore be shipped under cooled conditions, the plugs should however never be frozen.

5.2 Long-term stability study

For the long-term stability study, units were stored at 4 °C and 18 °C for 0, 3, 6, 9 and 12 months (at each temperature). The reference temperature was set to 4 °C. 12 units per storage time were selected using a random stratified sampling scheme. From each time and temperature point, 6 units were measured by PFGE in duplicate. The measurements were performed under repeatability conditions, in a random sequence.

The obtained results were evaluated individually for each temperature.

The results of the long term stability measurements are shown Figure 3.

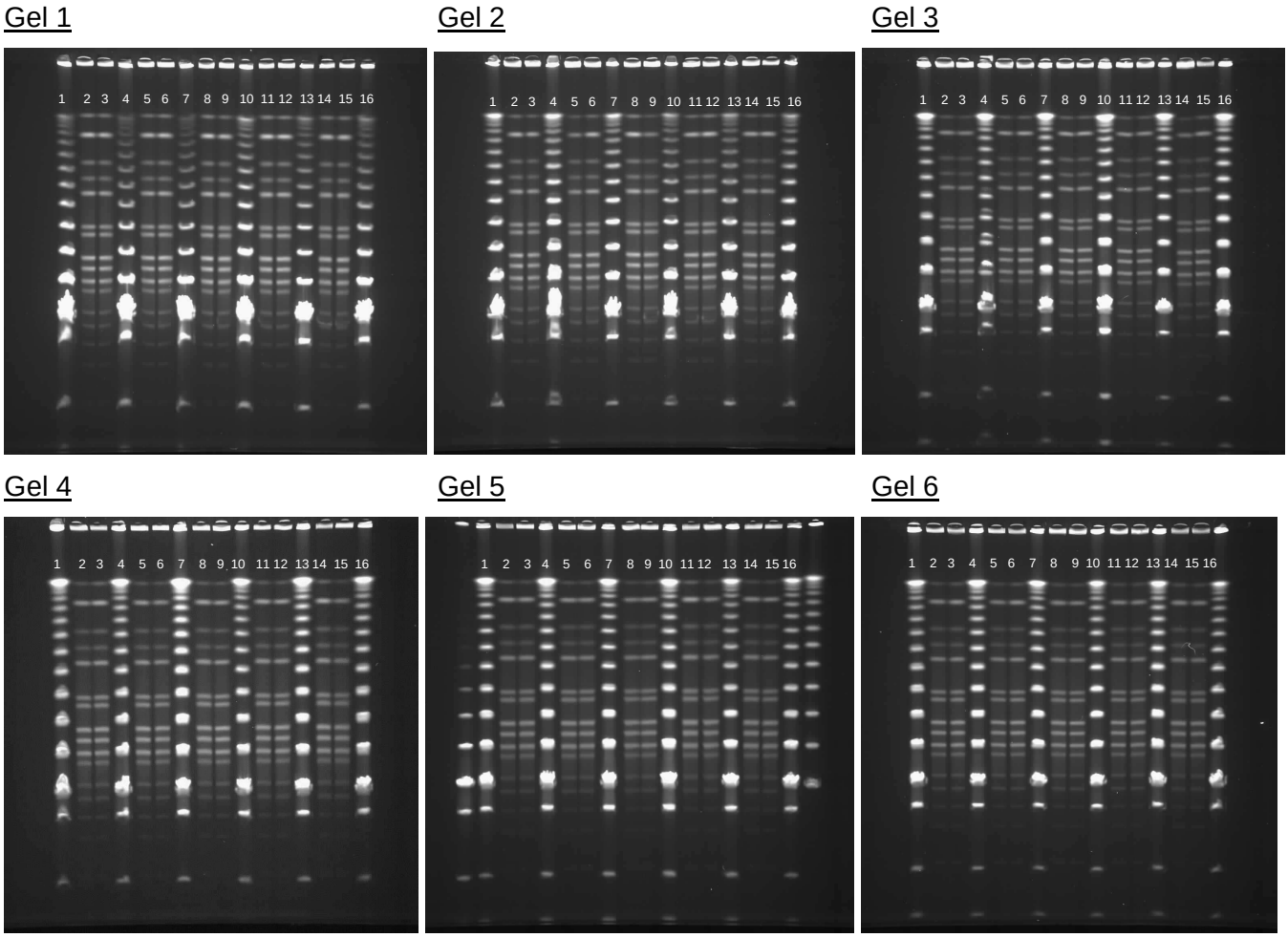

Figure 3: PFGE pattern of size markers and long-term stability samples, obtained by PFGE analysis as described in section 3.4. Lanes 1, 4, 7, 10, 13, 16: size marker D2291 and D2416 (Sigma). Lanes 2, 3, 5, 6, 8, 9, 11, 12, 14, 15 correspond to a series of sample numbers in the table in Annex B.

PFGE analysis of the long-term stability samples showed identical band patterns for all units stored at 4 ºC and 18 ºC. i.e. no bands were missing, no new bands appeared, no smears nor obvious differences in the resolution of the bans were observed.

It can be concluded that the batch is stable up to 18 ºC. It is recommended to store the material at 4 ºC.

After the certification campaign, the material will be subjected to IRMM's regular stability monitoring programme to control its further stability.

6 Characterisation

Material characterisation is the process of determining the property values of a reference material.

The material characterisation was based on an intercomparison of expert laboratories, i.e. the properties of the material were determined in different laboratories. Due to the nature of the analyte, all participants used a PFGE method for the measurements. This approach aims at randomisation of laboratory bias, which reduces the combined uncertainty.

6.1 Selection of participants

8 laboratories were selected based on criteria that comprised both technical competence and quality management aspects. Each participant was required to operate a quality system and to deliver evidence of its laboratory proficiency in the field of PFGE measurements by submitting results of the analysis of a PFGE test sample provided by IRMM. Having a formal accreditation was not mandatory, but meeting the requirements of ISO/IEC 17025 was obligatory.

6.2 Study setup

Each laboratory received 4 units of IRMM-313 and was requested to provide 8 independent results, 2 per unit. The units for material characterisation were selected using a random stratified sampling scheme and covered the whole batch. The sample preparations and measurements had to be spread over at least two days to ensure intermediate precision conditions.

6.3 Methods used

All laboratories were instructed to use the same analysis protocol, described in section 3.4.

The fragment sizes were established by comparing their migration distance to the migration distance of fragments of size markers Pulse Marker 0.1-200 kb (D2291; Sigma) and Pulse Marker 50-1,000 kb (D2416; Sigma) using a designated software. The size markers consist of restricted λ DNA fragments, complete λ DNA and λ DNA concatemers, all with exactly known fragment sizes.

All labs apart from 2 used BioNumerics software to assign fragment sizes, 1 lab used BioRAD FPQuest and 1 lab used Gene Tools from Syngene.

6.4 Evaluation of results

6.4.1 Technical evaluation

The characterisation campaign resulted in 8 datasets.

The obtained data were first checked for compliance with the analysis protocol and for their validity based on technical reasons. Compliance with the analysis protocol, sample preparations, spread of measurements over two days, and the analytical sequence were considered during the evaluation.

Deviations from the instructions were communicated by the laboratories.

Most deviations reported did not affect the validity of the dataset. E.g. staining with Gel Red stain instead of with ethidium bromide solution, the addition of Bovine Serum Albumin to the digestion mix, the use of a different comb size.

However lab 6 reported a deviation from the required temperature criteria. Therefore the dataset of lab 6 was rejected for technical reasons and the data was not considered for value assignment.

Additionally results of labs 2, 3 and 4 for fragments 15 and 16 were not considered as the size markers flanking these fragments were not visible and could therefore not be used for fragment size assignment.

All individual results of the participants are displayed in tabular form in Annex C.

6.4.2 Statistical evaluation

The accepted datasets were tested for normal distribution of dataset means using kurtosis/skewness tests and normal probability plots and were tested for outlying means using the Grubbs test and using the Cochran test for outlying standard deviations, both at a 99 % confidence level. Standard deviations within (s_{within}) and between ($s_{between}$) laboratories were calculated using one-way ANOVA.

The uncertainty related to the characterisation is estimated as the standard error of the mean of laboratory means.

The results of these evaluations are shown in Table 2.

Table 2: Statistical description of the accepted datasets for IRMM-313. *p*: number of technically valid datasets

IRMM-313 fragment number	*p*	Outliers		Normally distributed	Statistical parameters				
		Means	Variances		Mean [kb]	*s* [kb]	$s_{between}$ [kb]	s_{within} [kb]	u_{char} [kb]
1	7	No	No	Yes	571.7	33.6	45.9	7.8	12.7
2	7	No	Yes	Yes	458.8	2.6	3.2	3.9	1.0
3	7	No	No	Yes	351.7	3.2	4.3	2.3	1.2
4	7	No	No	Yes	303.0	3.1	4.3	1.6	1.2
5	7	No	No	Yes	263.2	2.5	3.4	1.5	0.9
6	7	Yes	No	No	188.2	1.6	2.2	1.1	0.6
7	7	No	No	Yes	173.2	1.7	2.4	0.9	0.7
8	7	No	Yes	Yes	131.1	1.9	2.7	1.2	0.7
9	7	No	Yes	Yes	114.4	1.5	2.1	1.3	0.6
10	7	No	Yes	Yes	95.5	1.9	2.5	1.6	0.7
11	7	No	Yes	Yes	81.2	2.2	3.0	1.5	0.8
12	7	No	No	Yes	54.9	2.9	4.0	1.0	1.1
13	7	No	No	Yes	40.7	2.1	2.9	0.8	0.8
14	7	No	No	Yes	25.4	1.8	2.5	0.5	0.7
15	4	No	n.a.	n.a.	17.6	0.3	0.3	0.3	0.1
16	4	No	n.a.	n.a.	10.9	0.4	0.6	0.2	0.2

The mean fragment size value of fragment 6 of laboratory 7 is an outlier at 99 % confidence level. The difference between the mean value of laboratory 7 and the other results is not covered by the measurement uncertainty of laboratory 7, there is thus evidence of a significant disagreement of results. However, closer investigation of results shows similar level of variance between the results of fragment 6 and other fragments. The significance of

the variance of results of fragment 6 can be attributed to the coincidental small s_{within} and $s_{between}$ of the remaining labs' results and can be seen as a statistical effect. The technical evaluation of results did not indicate any technical flaws in the method, there is no reason for discarding any of the results.

The mean fragment size value of fragment 6 is additionally flagged as not normally distributed at 99 % confidence level. A closer investigation reveals that the not normal distribution is due to the outlying result of laboratory 7. We consider this as an additional statistical effect and propose to retain the data set and assign a value.

The statistical evaluation flags outlying variances for several fragments. As all laboratories used the same PFGE method, this reflects the intrinsic variability of the method. As the measurement method was found technically sound, all results were retained.

7 Value Assignment

Certified values, indicative values and additional material information values were assigned.

Certified values are values that fulfil the highest standards of accuracy. Procedures at IRMM require generally pooling of not less than 6 datasets to assign certified values. Full uncertainty budgets in accordance with the 'Guide to the Expression of Uncertainty in Measurement' [3] were established. Certified values were assigned to fragments 2 to 13 which are within the working range of the size marker.

Indicative values are values where either the uncertainty is deemed too large or where too few independent datasets were available to allow certification. Uncertainties are evaluated according to the same rules as for certified values. Such values were assigned to fragments 14, 15 and 16 for having incomplete datasets. These values are assigned the same way as the certified values but are only indicative values and should only be used as such.

Additional material information refers to values that were obtained in the course of the study. For example, results reported from only one or two laboratories or in cases where individual measurement uncertainty is high, would fall under this category. Such value was assigned to fragment 1 for being outside the working range of the size markers. This value is assigned the same way as the certified values but is only additional material information and should only be used as such.

7.1 Certified values and their uncertainties

The unweighted mean of the means of the accepted datasets as shown in Table 2 was assigned as certified value for each fragment. The PFGE pattern can be seen in Figure 4.

The assigned uncertainty consists of the uncertainty related to characterisation, u_{char} (Section 6) only. No uncertainties were calculated for homogeneity and stability as they were only assessed qualitatively by visual inspection. This uncertainty was used to estimate the expanded uncertainty of the certified value (U_{CRM}) with a coverage factor k as:

$$U_{CRM} = k \cdot \sqrt{u_{char,rel}^2} \qquad \textbf{Equation 1}$$

u_{char} was estimated as described in Section 6.

Table 3: Certified values and their uncertainties for IRMM-313.

IRMM-313 fragment number	Certified value [kb]	$u_{char, rel}$ [%]	$U_{CRM, rel}$ [%]	U_{CRM} [kb] [1)]
2	458.8	0.2	0.4	2.0
3	351.7	0.3	0.7	2.4
4	303.0	0.4	0.8	2.3
5	263.2	0.4	0.7	1.9
6	188.2	0.3	0.6	1.2
7	173.2	0.4	0.8	1.3
8	131.1	0.6	1.1	1.5
9	114.4	0.5	1.0	1.2
10	95.5	0.7	1.5	1.4
11	81.2	1.0	2.0	1.7
12	54.9	2.0	3.9	2.2
13	40.7	1.9	3.9	1.6

[1)] The uncertainty is the expanded uncertainty with a coverage factor k = 2 corresponding to a level of confidence of about 95 % estimated in accordance with ISO/IEC Guide 98-3, Guide to the Expression of Uncertainty in Measurement (GUM:1995), ISO, 2008.

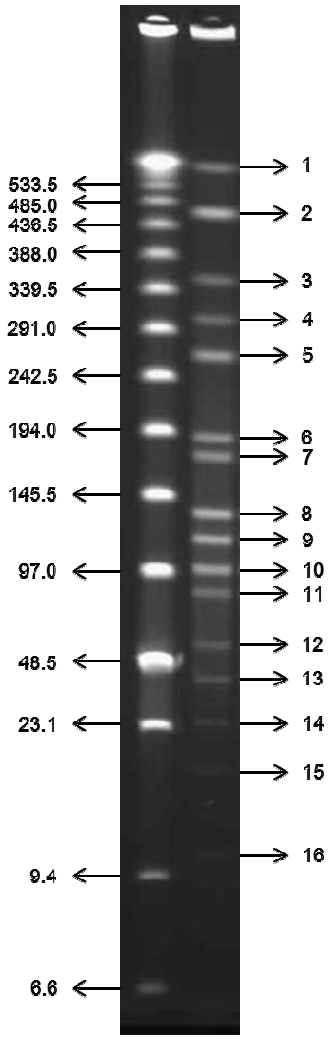

Figure 4: The left lane shows lambda DNA (cI857 ind 1 Sam7) fragments of size marker D2291 and D2416 with indicated fragment sizes [kb]. The right lane shows the certified PFGE pattern of the *Sma*I digested gDNA of IRMM-313.

7.2 Indicative values and their uncertainties

Indicative values were assigned for fragments 14, 15 and 16. For fragments 14 only 6 labs submitted complete datasets and for fragments 15 and 16 only 4 datasets were used for value assignment.

However, based on the statistical parameters of the obtained datasets, the results were regarded as sufficiently trustworthy to assign indicative values. Indicative values may not be used as certified values. The uncertainty budgets were set up as for the certified values and are listed together with the assigned values in Table .

Table 4: Indicative values and their uncertainties for IRMM-313

IRMM-313 fragment number	Indicative value [kb]	$u_{char, rel}$ [%]	$U_{CRM, rel}$ [%]	U_{CRM} [kb] [1)]
14	25.4	2.6	5.3	1.3
15	17.6	0.7	1.5	0.3
16	10.9	1.9	3.9	0.4

[1)] The uncertainty is the expanded uncertainty with a coverage factor k = 2 corresponding to a level of confidence of about 95 % estimated in accordance with ISO/IEC Guide 98-3, Guide to the Expression of Uncertainty in Measurement (GUM:1995), ISO, 2008.

7.3 Additional material information

The data provided in this section should be regarded as informative only and cannot be, in any case, used as certified or indicative value.

No certified value was assigned to fragment 1 as fragment 1 fell outside the working range of the size marker.

The uncertainty budgets were set up as for the certified values and are listed together with the assigned values in Table 5.

Table 5: Additional material information. Assigned values and their uncertainties.

IRMM-313 fragment number	Indicative value [kb]	$u_{char, rel}$ [%]	$U_{CRM, rel}$ [%]	U_{CRM} [kb] [1)]
1	571.7	2.2	4.4	25.4

[1)] The uncertainty is the expanded uncertainty with a coverage factor k = 2 corresponding to a level of confidence of about 95 % estimated in accordance with ISO/IEC Guide 98-3, Guide to the Expression of Uncertainty in Measurement (GUM:1995), ISO, 2008.

8 Metrological traceability and commutability

8.1 Metrological traceability

The DNA fragment sizes have been obtained by following the PFGE procedure described in section 3.

The fragment sizes were established by comparing their migration distance to the migration distance of fragments of size markers Pulse Marker 0.1-200 kb (D2291; Sigma) and Pulse Marker 50-1,000 kb (D2416; Sigma). The size markers consist of restricted λ DNA fragments, complete λ DNA and λ DNA concatemers, all with exactly known fragment sizes. Concatemers are linked DNA fragments, in this case complete λ DNA (cI857 ind 1 Sam7). The length of the λ DNA (cI857 ind 1 Sam7) molecule is known to be 48.5 kb and the length

of the concatemers was confirmed by sequencing the linkage sites between the λ DNA fragments. The length of the fragmented λ DNA was confirmed by mapping the restriction sites of the restriction enzyme cutting the λ DNA.

All laboratories used the size markers with known λ DNA (cI857 ind 1 Sam7) fragment sizes. This makes the individual results traceable to the λ DNA (cI857 ind 1 Sam7), a DNA molecule which is well characterised with a known length and sequence. As the assigned values are combinations of agreeing results individually traceable to λ DNA (cI857 ind 1 Sam7), the assigned fragment sizes themselves are traceable to λ DNA (cI857 ind 1 Sam7).

8.2 Commutability

There are various definitions expressing the concept. For instance, the CSLI Guideline C-53A [8] recommends the use of the following definition for the term *commutability*:

> "The equivalence of the mathematical relationships among the results of different measurement procedures for an RM and for representative samples of the type intended to be measured."

As IRMM-313 is analysed only by PFGE as would be real samples, commutability is not an issue. The analytical behaviour of IRMM-313 will be the same as for routine *Campylobacter* samples used in PFGE.

9 Instructions for use

9.1 Safety information

The material should be handled under BSL 2 conditions. The usual laboratory safety measures apply.

9.2 Storage conditions

The materials shall be stored at 4 °C.

Please note that the European Commission cannot be held responsible for changes that happen during storage of the material at the customer's premises, especially of opened vials.

9.3 Minimum sample intake

The minimum sample intake is ½ plug.

9.4 Use of the material

The main purpose of this material is to assess method performance, i.e. for checking accuracy of analytical results.

Restriction enzyme digestion

- Incubate ½ plug in 200 µL 1x RE buffer solution, as recommended by the manufacturer, at room temperature for 15 min.

- Remove the buffer using a pipette and add 200 µL RE mix, as recommended by the manufacturer, containing 40 units of *Sma*I. Mix by tapping gently.

- Incubate at 25 ºC for 4 h.

PFGE

- Prepare 0.5x TBE (44.5 mM Tris-Borate; 1 mM EDTA, pH 8.3) buffer from 5x TBE buffer by dilution in Type I water.

- Fill the PFGE chamber with sufficient 0.5x TBE buffer, remove air bubbles trapped in the tubing, set the cooler at 14 °C and circulate the buffer for 1 h before use.

- Prepare 150 mL 1 % SKG agarose in 0.5x TBE and keep at 50 ºC until use

- Take a 30 well comb. Place the plug pieces on the comb as far to the bottom of the teeth as possible and fix them onto the comb by pipetting a few drops 1 % SKG agarose around the plugs. Place an IRMM-313 plug piece on the outside lanes of each gel and at least next to every 4th unknown sample.

- Assemble the casting stand and place it on a levelling table. It is important that the casting stand is completely horizontal to have the same gel thickness all over the gel.

- put the comb, with the plugs attached in place.

- Pour in the 1 % SKG agarose making sure not to move the plugs. Remove any air bubbles. Allow the gel to solidify for 30 to 45 min.

- Carefully remove the comb from the gel.

- Seal the resulting holes with 1 % SKG agarose.

- Take the gel and the black plate out of the casting stand. Remove any agarose from the bottom of the plate.

- Place the plate with the gel in the electrophoresis chamber, be sure that it fits tightly in the frame. Close the chamber, remove air bubbles trapped in the tubing and check that the cooler is working.

- Start the run with running conditions:

Initial switch time: 0.5 s

Final switch time: 40 s

Gradient: 5 V/cm

Angle: 120º

- Let run for 22.5 h

- Stain the gel 30 min in 400 mL 1 µg/mL ethidium bromide in Type I water.

- De-stain for 2x 20 min in 400 mL Type I water.

- Photograph the gel over UV-source using a UV orange filter.

- Analyse the gel using a dedicated software.

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Annexes

Annex A: Process control - Sequencing results

1) *C. coli* CNET068 stock and IRMM-313 plug sequence obtained with *C. coli* specific primers

Match	Description	Max score	Total score	Query cover	E value	Max ident	Accession
1	C.coli ceuB, ceuC, ceuD, ceuE, OrfA, OrfB genes	946	946	100%	0.0	99%	X88849.1
2	Campylobacter coli ceuE gene for enterochelin ABC transporter, periplasmic enterochelin-binding protein, partial cds, strain: K168	778	778	100%	0.0	93%	AB608209.1
3	Campylobacter coli strain 312 lipoprotein (ceuE) gene, partial cds	693	693	70%	0.0	99%	FJ946065.1
4	Campylobacter coli strain 128 lipoprotein (ceuE) gene, partial cds	688	688	70%	0.0	99%	FJ946064.1
5	Campylobacter coli strain 72 lipoprotein (ceuE) gene, partial cds	688	688	70%	0.0	99%	FJ946063.1
6	Campylobacter coli strain 39 lipoprotein (ceuE) gene, partial cds	688	688	70%	0.0	99%	FJ946062.1

C.coli ceuB, ceuC, ceuD, ceuE, OrfA, OrfB genes
Sequence ID: emb|X88849.1|Length: 5580Number of Matches: 1
Related Information
Range 1: 2878 to 3412GenBankGraphics Next Match Previous Match First Match
Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand
946 bits(512)	0.0	530/538(99%)	3/538(0%)	Plus/Plus

Features:

```
Query  1     AAAATTCCCAAAAATCCTTCTAAAGTTGTGATTTTAGATCTTGGAATTTTAGATACTTTC  60
             |||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2878  AAAATTCCAAAAAATCCTTCTAAAGTTGTGATTTTAGATCTTGGAATTTTAGATACTTTC 2937

Query  61    CATGCCCTAAGACTTAACGATAAAGTTGCAGGAGTTCCAGCTAAAAACTTGCCAAAATAC 120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2938  CATGCCCTAAGACTTAACGATAAAGTTGCAGGAGTTCCAGCTAAAAACTTGCCAAAATAC 2997

Query  121   TTACAGCAATTTAAAGACAAGCCTAGTATAGGTGGAGTTCAACAAGTTGATTTTGAAGCT 180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2998  TTACAGCAATTTAAAGACAAGCCTAGTATAGGTGGAGTTCAACAAGTTGATTTTGAAGCT 3057

Query  181   ATTAATGCTTTAAAACCTGATCTTATTATCATTTCCGGACGCCAAAGCAAATTTTATGAA 240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3058  ATTAATGCTTTAAAACCTGATCTTATTATCATTTCCGGACGCCAAAGCAAATTTTATGAA 3117

Query  241   AAATTAAAAGAAATTGCTCCAACTATGTTTGTAGGACTTGATAATGCAAATTTCTTAAGC 300
             ||||| ||| |  ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3118  AAATTGAAA-A--TTGCTCCAACTATGTTTGTAGGACTTGATAATGCAAATTTCTTAAGC 3174

Query  301   TCTTTTGAAAACAATGTTTTAAGTGTTGCAAAACTTTATGGCTTAGAAAAAGAAGCTTCT 360
             |||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct  3175  TCTTTTGAAAACAATGTTTTAAGCGTTGCAAAACTTTATGGCTTAGAAAAAGAAGCTTCT 3234

Query  361   GAAAAAATTGCAGATATTAAAAATGAGATAGAACAAGCAAAAAGCATAGTAGATGAAGAT 420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3235  GAAAAAATTGCAGATATTAAAAATGAGATAGAACAAGCAAAAAGCATAGTAGATGAAGAT 3294
```

```
Query  421   AAAAAAGCTCTTATTGTTCTAACCAATTCTAACAAAATTTCCGCTTTTGGACCTCAATCT 480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3295  AAAAAAGCTCTTATTGTTCTAACCAATTCTAACAAAATTTCCGCTTTTGGACCTCAATCT 3354

Query  481   CGCTTTGGAATCATTCATGACGTTTTAGGAATCAATGCTGTGGATGAAAATGGAAAAG  538
             |||||||||||||||||||| |||||||||||||||||||||||||||| |||||
Sbjct  3355  CGCTTTGGAATCATTCATGATGTTTTAGGAATCAATGCTGTGGATGAAAATGTAAAAG  3412
```

2) *C. jejuni* CNET112 stock and IRMM-313 plug sequence obtained with *C.jejuni* specific primers

Match	Description	Max score	Total score	Query cover	E value	Max ident	Accession
1	Campylobacter jejuni subsp. jejuni M1, complete genome	1275	1275	98%	0.0	100%	CP001900.1
2	Campylobacter jejuni subsp. jejuni 81116, complete genome	1275	1275	98%	0.0	100%	CP000814.1
3	Campylobacter jejuni ceuE gene for enterochelin ABC transporter, periplasmic enterochelin-binding protein, partial cds, strain: CO2-200	1203	1203	98%	0.0	98%	AB608208.1
4	Campylobacter jejuni ceuE gene for enterochelin ABC transporter, periplasmic enterochelin-binding protein, partial cds, strain: CO2-132	1203	1203	98%	0.0	98%	AB608207.1
5	Campylobacter jejuni ceuE gene for enterochelin ABC transporter, periplasmic enterochelin-binding protein, partial cds, strain: K162	1203	1203	98%	0.0	98%	AB608203.1
6	Campylobacter jejuni ceuE gene for enterochelin ABC transporter, periplasmic enterochelin-binding protein, partial cds, strain: K62	1203	1203	98%	0.0	98%	AB608202.1
7	Campylobacter jejuni subsp. jejuni 81-176 probable enterochelin uptake periplasmi-binding protein (cj1355), hypothetical protein (cju30), and putative integral membrane protein (cj1356c) genes, complete cds	1203	1203	98%	0.0	98%	DQ493921.1
8	Campylobacter jejuni ceuE gene for enterochelin ABC transporter, periplasmic enterochelin-binding protein, partial cds, strain: 81-176	1197	1197	98%	0.0	98%	AB608204.1
9	Campylobacter jejuni subsp. jejuni ICDCCJ07001, complete genome	1197	1197	98%	0.0	98%	CP002029.1
10	Campylobacter jejuni subsp. jejuni 81-176, complete genome	1197	1197	98%	0.0	98%	CP000538.1
11	Campylobacter jejuni subsp. jejuni PT14, complete genome	1182	1182	98%	0.0	98%	CP003871.2
12	Campylobacter jejuni subsp. jejuni NCTC 11168-BN148 complete genome	1182	1182	98%	0.0	98%	HE978252.1
13	Campylobacter jejuni ceuE gene for enterochelin ABC transporter, periplasmic enterochelin-binding protein, partial cds, strain: ATCC 43432	1182	1182	98%	0.0	98%	AB608205.1
14	Campylobacter jejuni ceuE gene for enterochelin ABC transporter, periplasmic enterochelin-binding protein, partial cds, strain: K38	1182	1182	98%	0.0	98%	AB608201.1
15	Campylobacter jejuni ceuE gene for enterochelin ABC transporter, periplasmic enterochelin-binding protein, partial cds, strain: K24	1182	1182	98%	0.0	98%	AB608200.1
16	Campylobacter jejuni subsp. jejuni IA3902, complete genome	1182	1182	98%	0.0	98%	CP001876.1
17	Campylobacter jejuni subsp. jejuni NCTC 11168 complete genome	1182	1182	98%	0.0	98%	AL111168.1
18	C.jejuni binding protein gene	1177	1177	98%	0.0	98%	X82427.1
19	Campylobacter jejuni ceuE gene for enterochelin ABC transporter, periplasmic enterochelin-binding protein, partial cds, strain: CO2-127	1155	1155	98%	0.0	97%	AB608206.1
20	Campylobacter jejuni subsp. jejuni S3, complete genome	1153	1153	98%	0.0	97%	CP001960.1
21	Campylobacter jejuni RM1221, complete genome	1153	1153	98%	0.0	97%	CP000025.1
22	Campylobacter jejuni strain 447 lipoprotein (ceuE) gene, partial cds	987	987	76%	0.0	100%	FJ946073.1
23	Campylobacter jejuni strain 170 lipoprotein (ceuE) gene, partial cds	987	987	76%	0.0	100%	FJ946071.1
24	Campylobacter jejuni strain 57 lipoprotein (ceuE) gene, partial cds	987	987	76%	0.0	100%	FJ946069.1
25	Campylobacter jejuni strain 217 lipoprotein (ceuE) gene, partial cds	911	911	76%	0.0	98%	FJ946072.1
26	Campylobacter jejuni strain 85 lipoprotein (ceuE) gene, partial cds	911	911	76%	0.0	98%	FJ946070.1
27	Campylobacter jejuni strain 37 lipoprotein (ceuE) gene, partial cds	911	911	76%	0.0	98%	FJ946068.1

Match	Description	Max score	Total score	Query cover	E value	Max ident	Accession
28	Campylobacter jejuni strain 36 lipoprotein (ceuE) gene, partial cds	911	911	76%	0.0	98%	FJ946067.1
29	Campylobacter jejuni strain 4 lipoprotein (ceuE) gene, partial cds	911	911	76%	0.0	98%	FJ946066.1
30	Campylobacter jejuni subsp. doylei 269.97, complete genome	291	291	30%	8e-75	92%	CP000768.1
31	Campylobacter jejuni strain 81-176 putative integral membrane protein (Cj1356c) gene, complete cds; tRNA-Ser gene, complete sequence; and hypothetical protein gene, complete cds	243	243	19%	2e-60	99%	AY532172.2

Campylobacter jejuni subsp. jejuni M1, complete genome
Sequence ID: gb|CP001900.1|Length: 1616648Number of Matches: 1
Range 1: 1277732 to 1278421GenBankGraphics Next Match Previous Match First Match
Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand
1275 bits(690)	0.0	690/690(100%)	0/690(0%)	Plus/Plus

Features:
Enterochelin ABC transporter, periplasmic enterochelin-bi...

```
Query  6        aaaTCCCTAAAAATCCTTCTAAGGTAGTGATCTTAGATCTTGGAATTTTAGATACTTTTG 65
                |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1277732  AAATCCCTAAAAATCCTTCTAAGGTAGTGATCTTAGATCTTGGAATTTTAGATACTTTTG  1277791

Query  66       ATGCTTTAAAATTAAACGATAAAGTCGCGGGCGTTCCTGCTAAAAATTTACCAAAATACC  125
                |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1277792  ATGCTTTAAAATTAAACGATAAAGTCGCGGGCGTTCCTGCTAAAAATTTACCAAAATACC  1277851

Query  126      TACAACAATTTAAAAACAAACCTAGTGTAGGTGGAGTACAACAAGTTGATTTTGAAGCCA  185
                |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1277852  TACAACAATTTAAAAACAAACCTAGTGTAGGTGGAGTACAACAAGTTGATTTTGAAGCCA  1277911

Query  186      TTAATGCTTTAAAACCTGATCTTATCATCATTTCTGGACGCCAAAGTAAATTTTATGACA  245
                |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1277912  TTAATGCTTTAAAACCTGATCTTATCATCATTTCTGGACGCCAAAGTAAATTTTATGACA  1277971

Query  246      AATTAAAAGAAATAGCTCCAACTTTATTTGTAGGTCTTGATAATGCAAATTTTTTAAGCT  305
                |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1277972  AATTAAAAGAAATAGCTCCAACTTTATTTGTAGGTCTTGATAATGCAAATTTTTTAAGCT  1278031

Query  306      CTTTTGAAAACAATGTCTTAAGCGTTGCAAAACTTTATGGTTTAgaaaaagaagctttgg  365
                |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1278032  CTTTTGAAAACAATGTCTTAAGCGTTGCAAAACTTTATGGTTTAGAAAAAGAAGCTTTGG  1278091

Query  366      aaaaaatttcagatattaaaaatgaaattgaaaaagccaaaagcatagttgatgaagata  425
                |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1278092  AAAAAATTTCAGATATTAAAAATGAAATTGAAAAAGCCAAAAGCATAGTTGATGAAGATA  1278151

Query  426      aaaaagCTCTTATTATTCTTACAAATTCTAACAAAATTTCAGCCTTTGGCCCTCAATCTC  485
                |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1278152  AAAAAGCTCTTATTATTCTTACAAATTCTAACAAAATTTCAGCCTTTGGCCCTCAATCTC  1278211

Query  486      GCTTTGGGATTATTCACGACGTTTTAGGGATTAACGCGGTAGATGAGAATATAAAAGTAG  545
                |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1278212  GCTTTGGGATTATTCACGACGTTTTAGGGATTAACGCGGTAGATGAGAATATAAAAGTAG  1278271

Query  546      GCACTCACGGTAAAAGTATCAATTCTGAATTTATATTAGaaaaaaaTCCTGATTATATTT  605
                |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1278272  GCACTCACGGTAAAAGTATCAATTCTGAATTTATATTAGAAAAAAATCCTGATTATATTT  1278331
```

```
Query  606      TTGTTATTGATAGAAATATCATTGTAGGCAACAAAGAACGGGCTCAAGGCATACTCGATA  665
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1278332  TTGTTATTGATAGAAATATCATTGTAGGCAACAAAGAACGGGCTCAAGGCATACTCGATA  1278391

Query  666      ATGCGCTTGTCGCTAAAACCAAAGCAGCAC  695
                |||||||||||||||||||||||||||||
Sbjct  1278392  ATGCGCTTGTCGCTAAAACCAAAGCAGCAC  1278421
```

Annex B: Homogeneity and stability sample numbers

Homogeneity

Gel 1		Gel 2	
Lane no.	Sample no.	Lane no.	Sample no.
2	107	**2**	853
3	399	**3**	1120
5	730	**5**	1369
6	272	**6**	940
8	458	**8**	1220
9	730	**9**	1369
11	458	**11**	1220
12	399	**12**	1120
14	272	**14**	940
15	107	**15**	853

Short-term stability

	Gel 1			Gel 2			Gel 3		
Lane no.	Time [month]	T [°C]	Sample no.	Time [month]	T [°C]	Sample no.	Time [month]	T [°C]	Sample no.
2	**0**	**4**	013	**0**	**4**	512	**0**	**4**	1061
3	**4**	**18**	224	**4**	**18**	693	**4**	**18**	1189
5	**1**	**18**	122	**1**	**18**	567	**1**	**18**	1107
6	**2**	**18**	154	**2**	**18**	661	**2**	**18**	1136
8	**1**	**-70**	500	**1**	**18**	567	**1**	**-70**	1200
9	**1**	**18**	122	**0**	**4**	512	**1**	**18**	1107
11	**0**	**4**	013	**4**	**18**	693	**0**	**4**	1061
12	**1**	**-70**	500	**2**	**18**	661	**1**	**-70**	1200
14	**4**	**18**	224	**1**	**-70**	5	**4**	**18**	1189
15	**2**	**18**	154	**1**	**-70**	5	**2**	**18**	1136

Long-term stability

	Gel 1			Gel 2			Gel 3		
Lane no.	Time [month]	T [°C]	Sample no.	Time [month]	T [°C]	Sample no.	Time [month]	T [°C]	Sample no.
2	**0**	**18**	7	**0**	**18**	274	**0**	**18**	520
3	**12**	**18**	111	**12**	**18**	365	**12**	**18**	612
5	**6**	**18**	51	**6**	**18**	309	**6**	**18**	551
6	**9**	**18**	87	**9**	**18**	339	**9**	**18**	585
8	**3**	**18**	26	**3**	**18**	294	**3**	**18**	526
9	**6**	**18**	51	**6**	**18**	309	**6**	**18**	551
11	**0**	**18**	7	**0**	**18**	274	**0**	**18**	520
12	**3**	**18**	26	**3**	**18**	294	**3**	**18**	526
14	**12**	**18**	111	**12**	**18**	365	**12**	**18**	612
15	**9**	**18**	87	**9**	**18**	339	**9**	**18**	585

	Gel 4			Gel 5			Gel 6		
Lane no.	**Time [month]**	**T [ºC]**	**Sample no.**	**Time [month]**	**T [ºC]**	**Sample no.**	**Time [month]**	**T [ºC]**	**Sample no.**
2	**0**	**18**	775	**0**	**18**	1007	**0**	**18**	1260
3	**12**	**18**	868	**12**	**18**	1113	**12**	**18**	1355
5	**6**	**18**	819	**6**	**18**	1070	**6**	**18**	1325
6	**9**	**18**	829	**9**	**18**	1099	**9**	**18**	1346
8	**3**	**18**	793	**3**	**18**	1032	**3**	**18**	1298
9	**6**	**18**	819	**6**	**18**	1070	**6**	**18**	1325
11	**0**	**18**	775	**0**	**18**	1007	**0**	**18**	1260
12	**3**	**18**	793	**3**	**18**	1032	**3**	**18**	1298
14	**12**	**18**	868	**12**	**18**	1113	**12**	**18**	1355
15	**9**	**18**	829	**9**	**18**	1099	**9**	**18**	1346

Annex C: Summary of results of the characterisation study

		Fragment sizes [kb]															
Lab no.	**Sample no.**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
0	**1-1**	596.4	459.5	348.7	298.8	259.3	187.9	173.5	131.1	113.9	96.6	81.5	54.9	40.5	23.9	17.9	10.7
	1-2	574.4	454.0	346.4	296.8	257.6	186.4	171.8	130.0	112.9	94.8	79.8	54.5	40.0	23.8	17.6	10.7
	2-1	592.2	459.5	348.7	298.8	259.3	188.8	173.5	131.1	114.5	96.6	81.5	55.3	40.5	23.9	17.7	10.7
	2-2	574.4	456.3	347.8	298.1	258.7	187.3	172.7	130.7	113.6	95.5	80.5	54.9	40.5	23.8	17.7	10.7
	3-1	587.9	455.1	347.3	297.6	258.2	187.0	171.9	130.4	113.2	95.8	80.2	54.9	40.1	23.9	17.7	10.5
	3-2	574.4	456.3	346.4	298.1	258.7	187.3	172.7	130.7	113.6	95.5	80.5	54.9	40.0	23.8	17.6	10.6
	4-1	587.9	455.1	345.8	296.4	258.2	187.0	171.9	129.7	113.2	95.8	80.2	54.5	39.6	23.7	17.6	10.6
	4-2	574.4	454.0	344.9	296.8	257.6	187.3	171.8	130.0	112.9	94.8	79.8	54.5	40.0	23.5	17.6	10.6
	Mean	**582.8**	**456.2**	**347.0**	**297.7**	**258.4**	**187.4**	**172.5**	**130.5**	**113.5**	**95.7**	**80.5**	**54.8**	**40.1**	**23.8**	**17.7**	**10.6**
	s	**9.3**	**2.2**	**1.4**	**0.9**	**0.7**	**0.7**	**0.7**	**0.6**	**0.5**	**0.7**	**0.6**	**0.3**	**0.3**	**0.2**	**0.1**	**0.1**
1	**1-1**	601.9	461.7	349.2	299.7	260.3	189.0	174.4	133.1	117.0	97.8	83.1	54.6	39.4	24.4	17.4	10.5
	1-2	601.4	458.2	349.4	300.0	260.8	187.3	172.5	131.0	114.7	95.8	82.6	56.6	40.6	23.9	17.0	10.3
	2-1	601.0	465.8	350.6	301.1	261.5	188.2	173.8	132.5	116.3	97.2	82.6	54.3	39.1	24.3	17.4	10.5
	2-2	592.8	454.6	345.7	297.6	257.9	186.5	172.1	130.7	114.4	95.4	81.7	55.3	39.9	23.9	17.0	10.5
	3-1	620.6	461.6	353.4	304.7	264.1	188.3	173.6	132.8	116.7	97.4	82.4	53.5	38.7	24.2	17.3	10.6
	3-2	594.0	457.8	352.6	302.7	263.1	186.5	172.1	130.7	114.5	95.4	81.0	52.8	37.5	23.4	17.0	10.5
	4-1	617.1	458.9	351.7	303.7	263.0	187.9	173.9	132.7	116.6	97.8	82.3	52.8	37.9	24.4	17.5	10.5
	4-2	600.0	459.9	353.0	303.4	263.1	187.1	172.3	131.1	115.1	96.1	81.0	53.3	37.8	23.6	17.1	10.5
	Mean	**603.6**	**459.8**	**350.7**	**301.6**	**261.7**	**187.6**	**173.1**	**131.8**	**115.6**	**96.6**	**82.1**	**54.1**	**38.9**	**24.0**	**17.2**	**10.5**
	s	**10.0**	**3.3**	**2.6**	**2.4**	**2.0**	**0.9**	**0.9**	**1.0**	**1.1**	**1.0**	**0.8**	**1.3**	**1.1**	**0.4**	**0.2**	**0.1**
2	**1-1**	585.6	466.4	349.4	305.8	263.9	187.1	171.0	130.1	114.6	95.4	80.4	51.6	38.2	24.7	14.5	
	1-2	580.7	467.1	351.9	304.8	264.8	187.1	172.2	132.1	115.5	96.1	82.0	55.4	40.8	24.6	12.0	
	2-1	581.8	462.5	348.5	307.0	265.5	187.5	171.2	129.8	114.2	95.1	80.0	51.6	38.1	24.0	14.3	
	2-2	582.9	457.9	352.2	305.0	265.3	187.5	172.8	132.6	115.8	96.6	82.5	55.6	41.3	24.4	12.4	
	3-1	578.6	458.0	351.6	307.5	267.2	189.9	173.9	130.8	114.1	94.4	79.7	52.3	38.7	24.3	14.1	
	3-2	588.8	469.1	353.2	306.4	266.7	188.4	173.0	132.6	116.1	97.0	82.9	55.5	40.8	23.8	11.9	
	4-1	580.1	458.7	353.8	306.7	266.4	190.7	174.7	131.8	114.8	95.0	80.2	52.9	38.9	24.0	14.2	
	4-2	586.7	467.3	352.5	305.1	265.4	187.4	172.1	131.7	115.4	96.2	82.1	54.7	40.0	23.3	11.9	
	Mean	**583.2**	**463.4**	**351.6**	**306.0**	**265.7**	**188.2**	**172.6**	**131.4**	**115.1**	**95.7**	**81.2**	**53.7**	**39.6**	**24.1**	**13.2**	
	s	**3.6**	**4.7**	**1.8**	**1.0**	**1.1**	**1.4**	**1.3**	**1.1**	**0.7**	**0.9**	**1.3**	**1.8**	**1.3**	**0.5**	**1.2**	
3	**1-1**	542.0	458.3	353.1	308.0	266.6	186.1	174.0	121.0	111.5	90.6	76.2	50.2	38.0	26.3	17.8	
	1-2	566.0	471.2	355.1	302.8	263.6	186.3	174.1	130.6	112.7	92.3	77.6	51.2	38.6			
	2-1	538.4	456.0	363.4	305.3	265.1	186.3	173.3	129.4	110.9	90.4	76.5	48.8	37.0	25.6	17.7	
	2-2	567.0	471.2	366.0	302.8	264.3	186.2	174.1	130.8	112.9	92.3	77.5	51.5	38.5			
	3-1	529.5	450.1	349.5	304.2	263.0	187.3	172.3	129.0	111.1	91.2	76.4	50.1	37.9	26.2		
	3-2	541.3	458.6	351.4	302.6	264.2	187.6	172.9	130.5	112.5	92.6	76.0	51.0	39.1			
	4-1	525.8	449.4	348.1	301.3	261.2	186.5	171.4	129.3	112.5	92.9	77.9	50.6	38.5	27.0	18.2	
	4-2	540.8	459.4	352.6	303.1	265.2	187.8	172.6	129.4	111.6	91.1	77.8	50.8	38.7			
	Mean	**543.8**	**459.3**	**354.9**	**303.8**	**264.2**	**186.8**	**173.1**	**128.7**	**112.0**	**91.7**	**77.0**	**50.5**	**38.3**	**26.3**	**17.9**	
	s	**15.2**	**8.3**	**6.5**	**2.1**	**1.6**	**0.7**	**1.0**	**3.2**	**0.8**	**1.0**	**0.8**	**0.8**	**0.7**	**0.6**	**0.2**	
4	**1-1**	559.6	456.8	352.0	304.1	264.6	186.7	171.5	130.5	115.1	96.8	83.1	56.6	43.9	30.7	20.7	10.1
	1-2	562.9	459.7	349.1	304.0	263.8	185.1	170.1	127.3	110.0	90.4	76.4	50.9	38.8	26.7	17.9	8.5
	2-1	557.3	458.2	349.1	302.5	264.2	185.7	171.1	130.4	113.0	94.2	80.1	55.4	42.9	30.2	20.2	9.8
	2-2	561.8	461.3	347.9	303.5	263.2	184.5	169.7	126.6	108.8	89.1	74.8	50.2	38.3	26.3	17.5	8.5
	3-1	557.1	459.4	348.7	302.0	262.8	185.0	168.9	128.0	114.6	97.0	82.8	55.4	42.5	29.6	19.5	9.6
	3-2	556.5	457.9	353.5	303.5	264.5	190.1	174.4	132.4	116.9	98.9	84.3	56.0	42.7	29.8	20.0	9.8
	4-1	559.9	456.0	348.0	301.1	263.6	184.7	169.0	127.6	113.0	93.6	79.8	52.9	40.6	28.0	18.4	9.1
	4-2	558.5	457.9	354.7	304.6	265.4	190.9	174.9	132.1	116.2	97.7	83.2	55.0	42.1	29.2	19.8	9.6
	Mean	**559.2**	**458.4**	**350.4**	**303.2**	**264.0**	**186.6**	**171.2**	**129.3**	**113.4**	**94.7**	**80.5**	**54.0**	**41.5**	**28.8**	**19.2**	**9.4**
	s	**2.3**	**1.7**	**2.7**	**1.2**	**0.8**	**2.5**	**2.3**	**2.3**	**2.9**	**3.6**	**3.5**	**2.4**	**2.0**	**1.6**	**1.2**	**0.6**

		Fragment sizes [kb]															
Lab no.	**Sample no.**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
5	**1-1**	515.6	454.8	351.2	302.2	262.9	188.1	173.6	129.5	112.0	93.3	81.1	58.0	42.1	25.5	19.1	11.8
	1-2	519.1	455.2	351.9	303.6	265.2	189.2	174.4	132.3	116.3	98.3	84.4	58.0	42.2	24.8	17.5	11.4
	2-1	513.5	452.0	356.9	299.5	260.8	186.5	171.8	129.0	112.3	94.5	82.3	58.7	43.7	26.1	18.4	11.8
	2-2	516.5	453.4	350.6	302.1	263.9	188.7	173.9	131.9	115.7	97.8	84.0	57.3	41.4	24.5	17.6	11.4
	3-1	523.1	458.6	353.1	301.4	262.4	187.7	172.7	130.5	115.1	97.5	84.1	57.5	41.1	24.1	17.1	11.3
	3-2	519.8	455.9	353.3	303.7	264.7	188.5	174.1	132.7	116.6	98.5	84.7	57.0	42.1	24.9	17.6	11.4
	4-1	523.5	458.6	351.6	301.7	261.4	187.6	172.9	130.5	114.3	96.1	82.7	56.9	41.0	24.2	17.5	10.9
	4-2	516.0	453.1	352.1	302.7	263.8	187.6	172.7	131.1	115.5	97.8	84.1	57.3	41.5	24.7	17.5	11.5
	Mean	**518.4**	**455.2**	**352.6**	**302.1**	**263.1**	**188.0**	**173.3**	**130.9**	**114.7**	**96.7**	**83.4**	**57.6**	**41.9**	**24.9**	**17.8**	**11.4**
	s	**3.6**	**2.4**	**2.0**	**1.3**	**1.5**	**0.8**	**0.9**	**1.3**	**1.7**	**1.9**	**1.3**	**0.6**	**0.9**	**0.7**	**0.6**	**0.3**
7	**1-1**	633.9	469.7	359.7	309.8	264.9	190.6	176.1	134.7	117.6	97.2	83.5	58.2	42.8	24.9	17.1	10.9
	1-2	586.9	448.3	353.6	303.6	262.7	192.3	177.5	135.5	116.3	97.1	84.0	60.3	46.2	26.5	18.0	11.1
	2-1	618.4	462.6	357.2	306.3	264.7	191.8	177.2	135.2	117.8	98.4	84.1	58.2	42.0	24.6	17.1	10.8
	2-2	594.1	450.4	354.9	305.7	264.3	191.5	177.0	135.1	116.2	97.5	83.4	59.9	46.0	26.2	18.0	11.1
	3-1	625.0	465.2	358.8	306.7	266.4	191.9	177.4	134.2	116.4	96.7	83.0	59.3	42.6	24.7	17.5	10.8
	3-2	618.0	455.8	359.6	308.5	266.1	191.0	175.9	134.9	115.4	97.7	83.8	60.0	45.9	26.5	18.2	11.3
	4-1	628.7	466.3	358.1	307.2	266.1	192.5	177.2	134.3	116.4	97.3	83.1	58.8	43.2	24.6	17.3	10.9
	4-2	604.9	456.3	358.4	308.2	265.9	190.8	176.3	133.9	115.4	96.7	83.4	60.0	46.3	26.6	18.2	11.2
	Mean	**613.7**	**459.3**	**357.5**	**307.0**	**265.1**	**191.6**	**176.8**	**134.7**	**116.4**	**97.3**	**83.5**	**59.3**	**44.4**	**25.6**	**17.7**	**11.0**
	s	**16.8**	**7.8**	**2.2**	**1.9**	**1.2**	**0.7**	**0.6**	**0.6**	**0.9**	**0.6**	**0.4**	**0.9**	**1.9**	**1.0**	**0.5**	**0.2**

European Commission
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Title: **Certification of a reference material of Campylobacter coli and jejuni (CNET068 and CNET112) agarose plugs for PFGE: IRMM-313**

Author(s): N. Meeus, J. Simonet, H. Schimmel

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Abstract

This report describes the production of IRMM-313, a Campylobacter coli and jenuni gDNA material certified for the size of the DNA fragments obtained by enzymatic restriction and Pulsed Field Gel Electrophoresis (PFGE). The material was produced following ISO Guide 34:2009.
The CRM was produced from cultures of Campylobacter coli CNET068 and jejuni CNET112 which were pooled and processed into agarose plugs suited for PFGE. The bacterial cells were lysed as to release the gDNA within the plug.
Between unit-homogeneity and stability during dispatch and storage were assessed in accordance with ISO Guide 35:2006.
The material was characterised by an intercomparison among laboratories of demonstrated competence and complying to ISO/IEC 17025. Technically invalid results were removed but no outlier was eliminated on statistical grounds only. The certified values were obtained by PFGE.
Uncertainties of the certified values were calculated in compliance with the Guide to the Expression of Uncertainty in Measurement (GUM).
The material is intended for quality control and assessment of method performance. As any reference material, it can also be used for control charts or validation studies.
The CRM is available in plastic screw cap vials containing 1 plug suspended in Tris EDTA buffer solution. The minimum amount of sample recommended to be used is ½ plug.

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